Available online at www.sciencedirect.com

ScienceDirect

Neuromuscular Disorders 29 (2019) 487–496

www.elsevier.com/locate/nmd

Inflammation-induced fibrosis in skeletal muscle of female carriers of Duchenne muscular dystrophy

Corinna Preuße[a,b,#], Arpad von Moers[b,#], Heike Kölbel[c], Debora Pehl[a], Hans-Hilmar Goebel[a,d], Ulrike Schara[c,§], Werner Stenzel[a,§,*]

[a] *Department of Neuropathology, Charité - Universitätsmedizin, Charité Campus Mitte, Corporate Member of Freie Universität Berlin, Humboldt-Universität zu Berlin, and Berlin Institute of Health (BIH), Berlin, Germany*

[b] *Department of Paediatrics and Neuropaediatrics, DRK Klinikum Westend, Berlin, Germany*

[c] *Department of Neuropaediatrics, Neuromuscular Centre, Universitätsklinikum, Essen, Germany*

[d] *Department of Neuropathology, Universitätsmedizin Mainz, Mainz, Germany*

Received 20 December 2018; received in revised form 4 March 2019; accepted 3 May 2019

Abstract

Female carriers of *DMD* gene mutations may be symptomatic and show variable skeletal as well as cardiac muscle symptoms. Skeletal muscle can exhibit morphological alterations. However, inflammatory, degenerative and fibrotic changes as seen in Duchenne boys have not been specifically analysed yet, so we addressed the question whether skeletal muscle of female carriers show such alterations. Thirteen carriers with an age range of 3 to 50 years were studied retrospectively. Five out of 13 women had clinically affected relatives. Clinically, most women showed mild muscle weakness, while the CK levels were increased in nine of them. Histomorphological analyses highlighted the typical mosaic pattern of dystrophin-positive and -negative fibres. Regenerating fibres were diffusely scattered and focally pronounced, while endo- and perimysial fibrosis was a variable but constant feature. Infiltration of $CD206^{+}TGF\beta^{+}$ macrophages and scattered T cells was noted in the endomysium. *TGFb* and *CCL18*, were significantly increased. However, gene expression of markers involved in Th1/Th2 immunity did not reach statistical significance compared to non-diseased controls. In summary, skeletal muscle of clinically manifest female *DMD* gene mutation carriers shows mild fibrosis and increased regeneration associated with endomysial $CD206^{+}TGF\beta^{+}$ and $STAT6^{+}$ macrophages, which are most likely involved in fibrotic remodelling.

Keywords: Female carriers; *DMD* gene; Dystrophin protein; Fibrosis; Inflammation, TGFβ.

1. Introduction

Duchenne muscular dystrophy (DMD) is an X-chromosomal recessively inherited disease, which affects boys from early childhood on. Loss or severe reduction of dystrophin protein induces a plethora of different physiological mechanisms finally leading to myofibre necrosis, atrophy, structural abnormalities, loss of homeostatic functions, and exhaustion of satellite cells. Over time, skeletal and cardiac muscles undergo structural remodelling with development of fibrosis and fatty tissue replacement. In addition, immuno-inflammatory processes within muscle tissue are active, mostly during early stages of disease [1,2].

The destruction of skeletal and cardiac muscle leads to progressive loss of mobility and subsequently, most boys become wheelchair-bound in their early teens. Over the last years, various therapies, mostly aiming at restoration of dystrophin expression, have been developed in model organisms and are currently employed in trials [3–5].

Females may also be affected by mutations of the *DMD* gene, however, the second healthy X-chromosome can compensate for this defect in most cases. Most female carriers of Duchenne gene mutations are asymptomatic, but 2–17% may manifest features of skeletal or cardiac muscle involvement [6–8]). Among asymptomatic carriers affected

* Corresponding author.
E-mail address: werner.stenzel@charite.de (W. Stenzel).
Shared first author.
§ Shared last author.

https://doi.org/10.1016/j.nmd.2019.05.003
0960-8966/

male relatives are often the first hint for their so-called 'gene carrier' status. Besides the development of skeletal muscle weakness as an isolated symptom, the carriers may also develop heart disease over time. This might lead to severe life-threatening problems; hence, thorough examination of skeletal and heart muscle is necessary for early diagnosis of this condition.

Typically, skeletal muscle exhibits a mosaic-like pattern of muscle fibres with reduced levels of dystrophin protein expression due to skewed X-inactivation with a less prominent myopathic/dystrophic pattern and less focal regeneration compared to that in affected boys.

In our study, we aimed at examining skeletal muscle biopsies of female carriers, focussing on immuno-inflammatory events and degenerative, fibrotic processes. Although rare, disease progression in affected females might worsen in skeletal and cardiac muscles and require treatment. Hence, we wanted to define key disease mechanisms, which could enable therapy.

2. Material and methods

2.1. Female carriers and biopsy specimens

We analysed cryopreserved skeletal muscle biopsy specimens from 13 female carriers with mutations in the *DMD* gene in comparison to non-diseased control biopsy specimens ($n=3$). Women showed disease specific symptoms ($n=11$), like manifest muscle weakness, elevated CK levels, cardiomyopathy and/or delayed developmental milestones. Two of them suffered from non-specific complaints such as fatigue or cramps, muscle pain [9,10]. In women without distinct symptoms, first- or second-degree relatives (e.g. son or brother) were affected by DMD; genetic analysis and muscle biopsy were performed to confirm a potential, yet unknown affection in these women. The available clinical information is summarised in Table 1. Non-diseased control (NDC) biopsies comprised three adult female patients with non-specific muscular complaints, which had led to muscle biopsy. Controls had normal clinical findings, normal serum CK, no laboratory evidence of systemic inflammation, and no morphological abnormalities in their skeletal muscle biopsies. All biopsies were obtained prior to any treatment. All specimens have been cryopreserved at −80 °C prior to diagnostic work-up. Informed consent was obtained from all patients. The study was reviewed and approved by the institutional ethics board of the Charité (No. EA1/204/11 and No. EA1/170/11).

2.2. Histology, enzyme histochemistry, and immunohistochemistry

Seven μm thick cryostat sections were stained by routine stains, enzymehistochemical preparations and immunohistochemical stains: Gömöri Trichrome, ATP-ase at pH 4.6, CCL18 (Lifespan, polyclonal, 1:100), CD4 (Zytomed, clone SP35, ready-to-use), CD8 (DAKO, clone C8/144B, 1:100), CD20 (DAKO, clone L26, 1:200), CD68 (DAKO, clone EBM11, 1:100), CD45 (DAKO, clone 2B11, 1:400), CD138 (DAKO, clone MI15, 1:30), CD206 (Acris, clone 7–450, 1:500), CollagenVI (Chemicon, clone VI-26, 1:200), C5b-9 (DAKO, clone aE11, 1:200), Dystrophin 1 (Novocastra, clone Dy4/6D3, 1:10), Dystrophin 2 (Novocastra, clone Dy8/6C5, 1:10), Dystrophin 3 (Novocastra, clone Dy10/12B2, 1:5), MHC class I (DAKO, clone W6/32 1:1000), MHC class II (DAKO, clone CR3/43, 1:100), neonatal MyHC (Novocastra, clone NB-MHCn, 1:20), STAT6 (R&D, clone 253906. 1:50), TGFβ (Abcam, clone 2Ar2, 1:30), and Utrophin (Novocastra, clone DRP3/20C5, 1:10).

Stains were performed using the iVIEW-Ventana DAB (diaminobenzidine)-Detection Kit (Ventana, Tucson, Arizona, 85755 USA). Appropriate biotinylated secondary antibodies were used, and visualization of the reaction product was carried out on a Benchmark XT immunostainer (Ventana) in a standardised manner. Omission of primary antibodies in control sections resulted in absence of any cellular labelling and demonstrated specificity of the primary antibodies. In addition, we used normal muscle tissues as negative control (or physiological internal control e.g. staining of MHC class I positivity of capillaries) for all reactions.

Immunostaining for fluorescent markers was performed in staining chambers after fixation in acetone for 10 min. The sections were then blocked with the appropriate serum (1:10 in PBS) dependent on the source of the secondary antibody, and incubated with the afore-mentioned primary antibodies over night at 4 °C or for 1 h at RT. After a washing step, the secondary antibody was added for 1 h. For double immune staining of CD206 and neoMyHC, CD206 and MHCII, CD206 and STAT6, and CD206 and TGFß, with the purpose to show co-localisation of two cellular structures, the above-mentioned protocol was performed using the first primary antibody and afterwards the same protocol was repeated with the second primary antibody and appropriate secondary antibodies. After a final washing step, the sections were aqueously mounted and stored at 4 °C.

2.3. Cell counts

For quantification of $CD4^+$ or $CD8^+$ T cells, $CD20^+$ B cells, $CD138^+$ plasma cells, and $CD68^+$ as well as $CD206^+$ macrophages, we performed cell counts of 10 high-power fields (one high-power field is defined as 0.16 mm^2) of each biopsy specimen. The average number of cells per high-power field per case is displayed.

2.4. Quantitative real-time polymerase chain reaction (qPCR)

RNA was extracted from whole muscle tissue using the trizol/chloroform method, according to the manufacturer's instruction (Invitrogen, Carlsbad, CA, USA). Thereafter, RNA was re-suspended and the concentration of total RNA was

Table 1.
Clinical data of female carriers with mutations in the *DMD* gene.

	age	CK-fold change	Symptoms	Family history	Type of mutation	Biopsy location	Classification
Female carrier							
Patient 01	6	33x	no specific complaints, general discomfort/fatigue, no objective muscle deficit, elevated transaminases, high CK levels	unknown	deletion	unknown	mfC
Patient 02	12	1,0	easily exhausted, muscle pain, headache, stomach pain, CK normal	unknown	deletion	M.quad	afC
Patient 03	32	increased	weakness, low resilience, cardiomyopathy, CK increased	affected relative	point mutation	M.delt	mfC
Patient 04	50	13,0	muscle weakness, muscle pain, reduced hearing capacity, increased CK level	empty	unknown	M.quad	mfC
Patient 05	12	66,0	mildly impaired dorsal extension of both feet, adipositas, contractures, secondary cardiomyopathy, high CK levels	affected relative	point mutation	M.quad	mfC
Patient 06	3	unknown	no specific complaints, general discomfort/fatigue	empty	point mutation	M.quad	afC
Patient 07	4	8,6	no specific complaints, general discomfort, CK mildly elevated, incomplete right bundle branch block	affected relative	unknown	M.quad	mfC
Patient 08	10	30,6	high CK level, no further symptoms known	affected relative	deletion	M.gast	mfC
Patient 09	8	20,0	developmental retardation since birth, reduced learning abilities*, mild bilateral contractures of hips, high CK level	empty	deletion	M.quad	mfC
Patient 10	17	13,6	muscle pain, delayed speaking abilities*, increased CK level	unknown	unknown	M.quad	Mfc
Patient 11	14	40,8	contractures, high CK level	affected relative	point mutation	M.quad	mfC
Patient 12	26	3,0	muscle pain, swelling of fingers, knees, muscle weakness while climbing stairs, general exhaustion, fatigue, dilatation of left ventricle, mildly elevated CK level	empty	deletion	M.quad	mfC
Patient 13	25	7,7	muscle weakness, muscle pain, mildly elevated CK level	empty	deletion	M.gast	mfC
Roundup characteristics female carrier ($n = 13$)							
	Mean age 17 years (range 3–50)	normal 8% >1-fold 31% >10-fold 45% unknown 15%	muscle weakness 31% muscle pain 38% contractures 23% cardiomyopathy 15% developmental deficiency* 15% non-specific complaints** 31% unknown 8%	aff. relative*** 38% empty 38% unknown 23%	point mutation 31% deletion 46% unknown 23%	M.delt 8% M.quad 70% M.gast 15%	
Roundup characteristics non-disease controls ($n = 3$)							
	41 years (range 26–57)	normal 100%	muscle pain 67% non-specific complaints** 33%	unknown 100%		M.delt 100%	

Remark: symptoms of NDC patients were subjective; we found no morphological changes or elevated blood parameter.
M.delt = M. deltoideus; M.quad = M. quadriceps; M.gast = M. gastrocnemius; mfC = manifest female carrier, afC = asymptomatic female carrier.
* first signs in young female carriers often were affected language development or motor deficiencies.
** e.g. headache, subjective reduced hearing capacity, fatigue.
*** Affection confirmed via muscle biopsy findings, muscle symptoms or increased CK values in first- or second-degree relatives.

photometrically determined with a TECAN fluorescence plate reader (Tecan, Männedorf, Switzerland). The RNA was reverse-transcribed using the High-Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA), according to the manufacturer's protocol, using 2 µg of total RNA per sample (as previously described [11]). For qPCR reactions, 2 ng of cDNA were used. All experiments were run as triplicates and each run contained the reference gene (*PGK1*) as internal control. The expression (meaning Ct value) of this reference gene was comparable in all analysed samples, including non-diseased controls, and unaffected by duration of the disease. To exclude loading differences and variations

between different runs all target genes were normalised to expression of *PGK1*. For analysis, the 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA) was used. Running conditions were as follows: 95 °C 0:20, 95 °C 0:01, 60 °C 0:20; 45 cycles (values above 40 cycles were defined as not expressed). The qPCR assay identification numbers are as follows:

CCL17: Hs00171074_m1; *CCL18*: HS00268113_m1; *CD301*: Hs00197107_m1; *cox2*: Hs00153133_m1; *IFNG*: Hs00989291_m1; *IL4*: Hs00929862_m1; *IL4R*: Hs00166237_m1; *IL5*: Hs01548712_g1; *IL10*: Hs00961622_m1; *IL12B*: Hs01011518_m1; *IL13*: Hs99999038_m1; *IL27R*: Hs00175472_m1; *MRC1* (CD206): Hs00267207_m1; *P4HA1*: Hs00990001_m1; *PGK1*: Hs99999906_m1; *STAT1*: Hs01013989_m1; *STAT2*: Hs01013123_m1; *STAT6*: Hs00598625_m1; *TGFB*: Hs00998133_m1; *TNFA*: Hs00174128_m1; *VEGFA*: Hs00900055_m1.

Data are represented as ΔCt values to show gene expression of the respective markers of female carriers, compared to non-diseased control patients' biopsies. All values are presented as scattered dot blots with means and standard deviation.

2.5. Statistics

Mann-Whitney U test was applied to analyse quantitative differences of mRNA transcripts. Kruskal-Wallis one-way ANOVA was applied to analyse cell counts, using Bonferroni correction of the post hoc tests. The level of significance was set at $p < 0.05$. Statistics were calculated with the GraphPad Prism 5.02 software (GraphPad Software, Inc., La Jolla, California, USA).

3. Results

3.1. Clinical data of female carriers with mutations in the DMD gene

We have analysed 13 female carriers, who had been identified as carriers of a mutated *DMD* gene, either due to elevated CK levels and affection of a male relative or due to manifest skeletal muscle weakness, and/or muscle cramps, most often exercise-dependent. DNA of most of the patients have been sequenced to determine the mutation inside the *DMD* gene. It was either a deletion detected (6/13), or a point mutation (4/13). In 3/13, we had no further information about the specific type of mutation.

Since we combined adult and juvenile patients, the mean age was 17 years, spanning 3-50 years of age. The CK levels were increased in all patients, but varied strongly between individual patients, with values often more than 10-fold increased (6/13). Clinically, these patients mostly complained about muscle weakness, muscle pain, and cardiac problems.

3.2. Female carriers show a mosaic pattern of dystrophin loss associated with fibrosis

To assess the involvement of skeletal muscle in disease progression we analysed general morphological alterations in female carriers. First, we demonstrated that the expression of dystrophin protein fragments was altered in all patients as expected, showing a mosaic pattern of dystrophin-positive and dystrophin-negative fibres. Since the locus of the mutation differed between the individuals, the binding for different dystrophin antibodies was affected accordingly: Either the C-terminal binding site, the N-terminal binding site, or the central rod domain was affected (exemplarily shown by staining with different dystrophin antibodies, Fig. 1A–C). Sarcolemmal utrophin expression with a focal character was present on muscle fibres in all patients, when compared to the normal expression, which is limited to capillaries. Focal utrophin staining demonstrates a compensating effect for the reduced dystrophin expression (Fig. 1D). Additional morphological changes comprised a widening of the perimysium and endomysium with increased fibrosis, as shown by Gömöri trichrome (Fig. 1E), EvG (not shown), and collagen VI stains (Fig. 3D). Analysis of slow and fast myosin heavy chain stains further showed altered distribution of type I and type II muscle fibres with grouping of and predominance of type I fibres reminiscent of (partial) fibre type disproportion (ATP-ase stain at pH 4.3 Fig. 1F).

Further stains for developmental myosin showed increased regeneration and focally immature fibres (developmental MyHC) (Fig. 1G). Regenerating fibres were found especially in areas of macrophage activity, as shown by double-staining of neonatal myosin and CD206 (Fig. 1H). These findings were found in all of the patients independently of age or clinical symptoms, demonstrating that these might be independent characteristics.

3.3. Leukocyte infiltration and inflammatory processes are mildly present in DMD carriers

Since muscle tissue of boys affected by DMD may show prominent infiltration by inflammatory cells, mainly comprising macrophages and T cells, we examined inflammatory processes in muscle of female carriers. However, fitting to the mild overall changes in morphology, we could only detect low numbers of inflammatory cells in our patients. Exemplarily, we show $CD68^+$ macrophages with diffuse distribution mostly in the perimysial areas, as well as single $CD45^+$ and $CD8^+$ T cells (Fig. 2A–C). As cell counts show, the main population is composed of $CD68^+$ macrophages, and only very few T cells are identified (Fig. 2D). Additionally, we stained for $CD20^+$ B cells and $CD138^+$ plasma cells, but could only detect single cells in two of our patients' muscle (Fig. 2D).

Two of our patients had no inflammation at all and might therefore have been biopsied at a time point of disease before

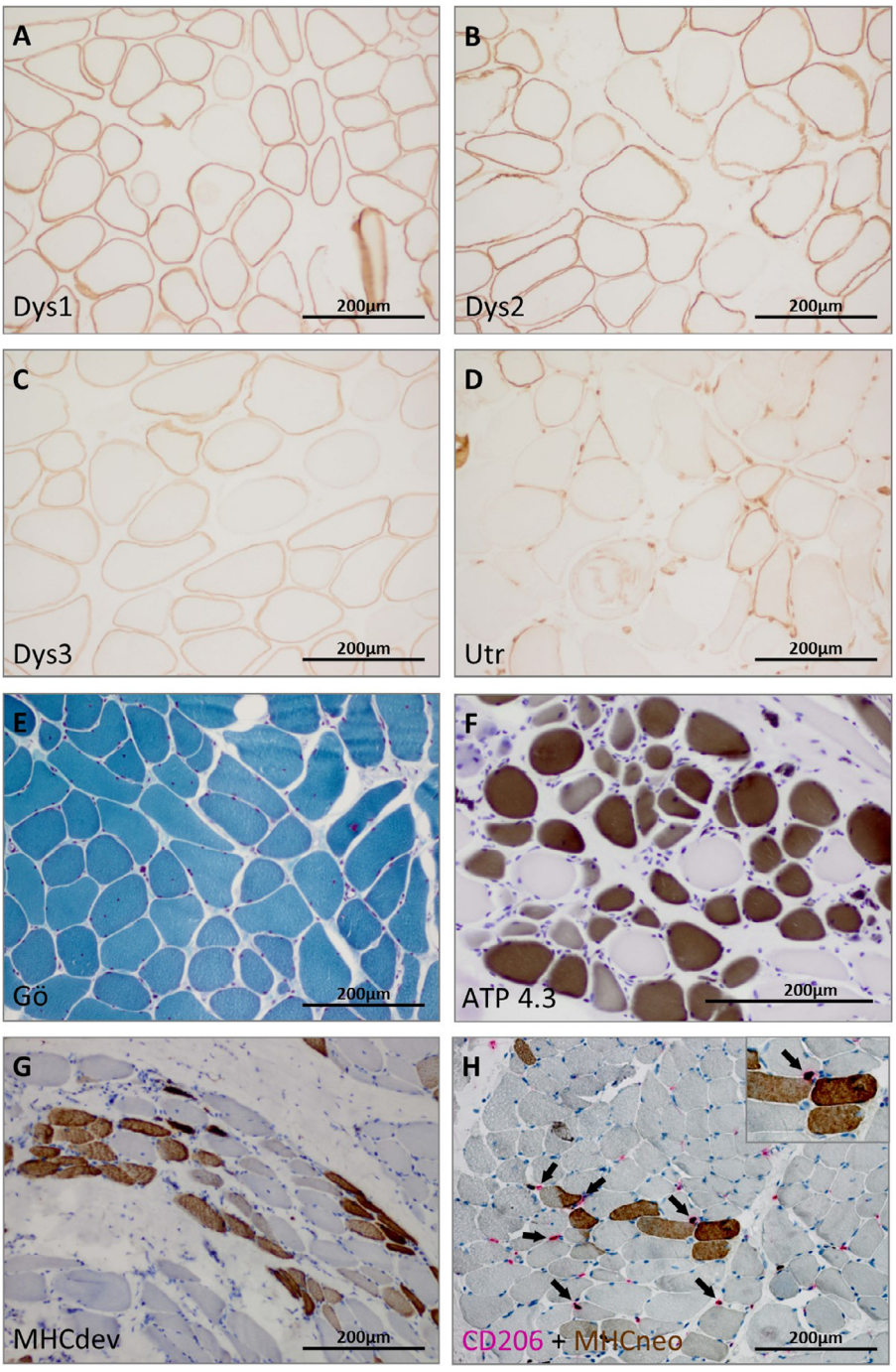

Fig. 1. Histological features of skeletal muscle of a female carrier of the *DMD* gene mutations
Dystrophin staining with Dys1 (A), Dys2 (B) and Dys3 (C) reveals reduced expression in all the stains. Expression of utrophin is normally found on capillaries, but is slightly increased on the sarcolemma in this female carrier (D). Staining with Gömöri trichrome shows some enlargement of the perimysium and endomysium in some areas of the muscle (E). Distribution of darkly stained type 1 and lighter stained type 2 fibres is changed from a homogenous checkerboard distribution to clustering of mainly type I fibres, shown by ATP-ase at pH 4.3 (F).
Expression of developmental MyHC demonstrates immaturity of muscle fibres (G) and double-staining of neonatal MyHC and CD206 highlights proximity of macrophages and regenerating fibres (H, and inset).

inflammatory alterations had developed or in a muscle that was not affected.

In addition, sarcolemmal staining of MHC class I molecules was found on about 50% of muscle fibres (Fig. 2E), and decoration of the sarcolemma with the complement factor C5b-9 could also be detected on some of the fibres (Fig. 2F).

Gene expression of molecular markers involved in Th1- and Th2-mediated immune reactions such as *IFNG*, *IL27R*, *IL10*, *IL4*, *IL13*, *IL4R* and *IL5* was not altered in

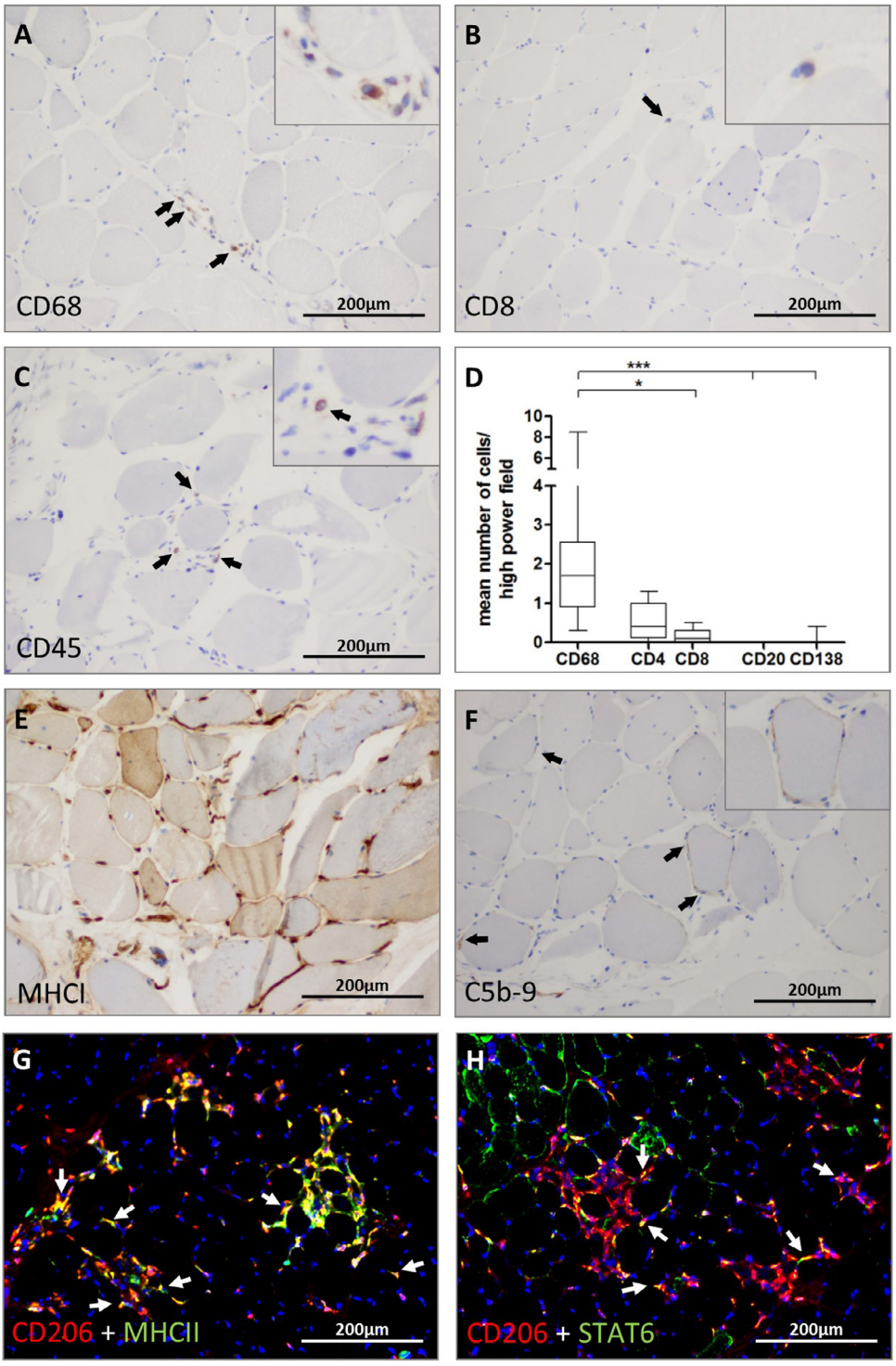

Fig. 2. Immune-mechanisms in female carriers' muscle parenchyma

Overall, invasion of the skeletal muscle by immune cells was mild in our cohort of patients. The predominant cells were $CD68^{+}$ macrophages (A), while only few T cells were detected (CD8; CD4 not shown: B). Leukocytes were also demonstrated with CD45 stains (C). Cell counts confirmed the histological analysis, and demonstrate that only single B cells and plasma cells are present (D). Sarcolemmal expression of MHC class I was upregulated focally on muscle fibres (E), while upregulation of the complement system was demonstrated by C5b-9 expression on some of the fibres (F). Co-expression of MHC class II (green) with CD206 (red) (G) as well as co-expression of STAT6 (green) with CD206 (red) (H) demonstrates polarisation of endomysial macrophages towards a pro-fibrotic phenotype.

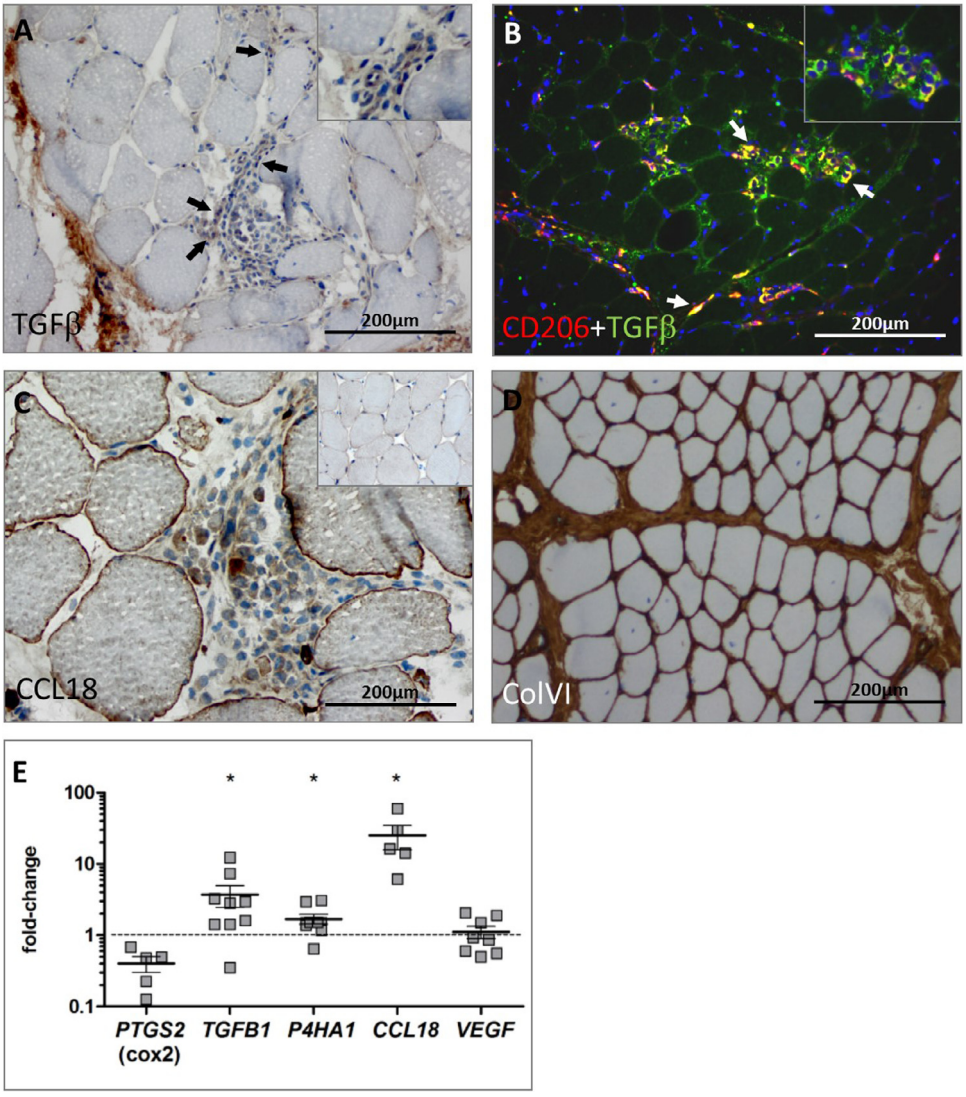

Fig. 3. Development of fibrosis in skeletal muscles of female carriers
Expression of TGFβ was found on some of the immune cells (A). Co-expression of TGFβ (green) with CD206 (red) shows that these cells were pro-fibrotic macrophages (B). CCL18 was upregulated on muscle fibres in female carriers (C) compared to NDCs (C inset). Collagen VI stained the basal lamina of all muscle fibres and the enlarged perimysium (D). Further qPCR analyses showed significantly increased expression of the fibrosis-associated genes *TGFB*, *P4HA1* and *CCL18* in comparison to female NDCs (E).

female carriers in comparison to NDCs (suppl. Fig. 1A, C). Since macrophages were the prevalent cell population, markers driving functional activation of macrophages were also analysed. We therefore tested gene expression for a more pro-inflammatory phenotype like *IL12*, *TNFA*, *STAT1*, *STAT2*, as well as markers for an alternative activation, such as *MRC1* (CD206), *CD301*, *STAT6* or *CCL17*. The transcription factor *STAT2* was significantly elevated in comparison to NDCs, as well as *CD301*, while all other markers were expressed at a level comparable to NDCs (suppl. Fig. 1B, D). We further characterised the macrophages on the protein level, demonstrating co-expression of CD206 with MHC class II as well as with STAT6 (Fig. 2G, H), complementing our results on the molecular level.

3.4. Fibrotic tissue remodelling is mild, but consistently present in all patients

Histological staining for TGFβ highlighted some of the immune cells (Fig. 3A), which were mostly CD206$^+$ macrophages (Fig. 3B), while collagen VI strongly stained the basal lamina and especially the strongly increased endo-and perimysial fibrous tissue (Fig. 3D). CCL18, which as a macrophage-derived pro-fibrotic chemokine was of particular interest, decorated most of the muscle cells of female carriers in comparison to NDCs, where CCL18 staining did not show any positivity (Fig. 3C NDC = inset). Similar to the inflammatory alterations, some patient's muscle showed stronger expression of CCL18 than others. To

further examine these changes on a molecular level, we isolated RNA from whole muscle tissue to determine the expression of markers associated with fibrosis. Here we could demonstrate that the expression of *TGFB* and *P4HA1* (Prolyl 4-Hydroxylase), which encode key molecules/enzymes of collagen synthesis, was significantly increased in female carriers when compared to NDCs (Fig. 3E). This was also true for *CCL18* in most of the patients, where levels were significantly increased.

4. Discussion

In this manuscript, we show that muscle biopsies of female carriers of a mutated *DMD* gene show morphological abnormalities in addition to the mosaic pattern of dystrophin protein loss. These consist of increased endo- and perimysial fibrosis, presence of $CD206^{+}TGF\beta^{+}$ and $CD206^{+}STAT6^{+}$ macrophages in the endo-and perimysium, and increased gene expression of molecules associated with fibrosis like *CCL18*, *PTGS2*, *TGFB* and *P4HA1*. These results indicate that fibrosis occurs during disease progression, and that it is likely driven by specifically activated endomysial macrophages. However, fibrosis was relatively mild. We did not observe any necrosis, myophagocytosis and no fatty replacement in any of the biopsies, all of which are morphological hallmarks in DMD boys.

Inflammation of skeletal muscle can be assumed in the case of sarcolemmal upregulation of MHC class I of myofibres, which are not in the process of regeneration. This is regularly the case in inflammatory myopathies (IIM) [12–16]. We were also able to show sarcolemmal complement deposition, which is another feature in IIMs, and which can occur in certain dystrophies as well (e.g. dysferlinopathies, anoctaminopathies, FSHD, laminopathies, etc.) [17]. The main population of inflammatory cells in the endo- and perimysium consisted of macrophages, and they were significantly more abundant as compared to non-diseased control subjects, which did harbour only occasional macrophages in their muscle. We have recently shown in skeletal muscle biopsies of patients with immune-mediated necrotizing myopathies (IMNM), that macrophages can have different subtypes and either express iNOS or CD206 [18]. $iNOS^{+}CD68^{+}$ macrophages in IMNM were identified in active myophagocytosis and within hyalinised early necrotic fibres, while $CD206^{+}$macrophages were found in the endomysium with diffuse distribution [18]. Here, in skeletal muscle biopsies of FCs we show that $CD206^{+}$ macrophages co-express $TGF\beta$ and can be identified close to foci of regeneration, which are neonatal MyHC-positive.

These types of macrophages have been shown to be involved in tissue remodelling and fibrosis in the lung of patients and of mice [19–21]. They are considered to be activated in a way, which is at variance with the classical pathway via $IFN\gamma$ and other Th1 cytokines and were initially called ‘alternatively activated’ [22]. However, it has become apparent that macrophages can be activated in many different ways, and that the spectrum is much larger than initially reported [22–25]. In our present work, we conclusively highlight presence of specifically activated macrophages closely interacting with foci of regeneration and increased endomysial collagen VI^{+} fibrosis. Interstitial fibrosis is accompanied by increased gene expression of prolin (*P4HA1*) and associated molecules of the arginine metabolism and part of the so-called ‘alternative activation’ of macrophages shown in pulmonary fibrosis [19,25–29]. Similar activation of the above mentioned molecular pathways was analysed in *mdx* mice by others [20]. It could be shown that reduction of arginine leads to reduced fibrosis, while dietary supplementation with arginine increased cardiac fibrosis [30]. These findings might be due to the affected balance between different macrophage subtypes, since distinct subpopulations can promote muscle injury or repair [20,31]. In addition, suppression of $IFN\gamma$ e.g. by Tregs, which are increased in *mdx* mice and Duchenne patients, and subsequent promotion of alternatively activated macrophages reduced muscle damage and improved motor performance [32,33].

Although in affected DMD males, the detailed analysis of the involved immune cells is still ongoing, new insights indicate that they have increased numbers of $CD8^{+}$ T cells in the peripheral blood, which directly correlates with results of quantitative muscle testing [34]. This finding is supported by the effect of prednisone in Duchenne patients, which reduced numbers of $CD8^{+}$ T cells and concomitantly improved muscle strength [35]. Even if further studies are needed, one feature in DMD patients is commonly accepted, that is upregulation of *TGFB* gene expression, which is what we also found in our female carriers. In this context, it might be interesting to further study the correlation of *TGFB* expression and disease state/progression, since various studies have shown involvement of TGFß in cardiac remodelling, cardiac fibrosis, and heart failure [36,37]. Furthermore, it was shown that therapies inhibiting the TGFß signalling pathways are beneficial in preventing cardiac fibrosis and heart failure [38,39]. However, despite the fact that some anti-TGF therapies already exist and might be a potential therapeutic approach, until now no standard anti-cytokine therapy for heart failure exists [40].

Studies about the development of muscle pathology in female carriers are scarce, and published results reflect mostly individual cases. However, MRI studies showing increased fibrosis in carriers [41], and H&E and dystrophin stains of muscle sections [42–44] are in line with our findings, as variation in fibre size, centralized nuclei, and inflammatory cell infiltration were signs of muscle affection. Hence, our results significantly expand these data and add more insights into disease mechanisms.

From a practical point of view, it may be important to follow manifest female carriers over the years with certain non-invasive and ideally with standardised techniques such as clinical assessment of muscle force, MRI and/or echocardiography of the heart and skeletal muscle to determine disease progression and eventually apply therapeutic measures.

Acknowledgement

The authors thank Sylvia Stefaniak, Svantje Hertel, Sabrina Forrell and Petra Matylewski for technical assistance.

Supplementary material

Supplementary material associated with this article can be found in the online version, at doi:10.1016/j.nmd.2019.05.003.

References

[1] Dumont NA, Wang YX, von Maltzahn J, Pasut A, Bentzinger CF, Brun CE, et al. Dystrophin expression in muscle stem cells regulates their polarity and asymmetric division. Nat Med 2015;21:1455–63.
[2] Klingler W, Jurkat-Rott K, Lehmann-Horn F, Schleip R. The role of fibrosis in Duchenne muscular dystrophy. Acta Myol 2012;31:184–95.
[3] Eghtesad S, Jhunjhunwala S, Little SR, Clemens PR. Effect of rapamycin on immunity induced by vector-mediated dystrophin expression in mdx skeletal muscle. Sci Rep 2012;2:2–7.
[4] Lu Q-L, Yokota T, Takeda S, Garcia L, Muntoni F, Partridge T. The status of exon skipping as a therapeutic approach to duchenne muscular dystrophy. Mol Ther 2011;19:9–15.
[5] Verhaart IEC, Heemskerk H, Karnaoukh TG, Kolfschoten IGM, Vroon A, van Ommen G-JB, et al. Prednisolone treatment does not interfere with 2'-O-methyl phosphorothioate antisense-mediated exon skipping in Duchenne muscular dystrophy. Hum Gene Ther 2012;23:262–73.
[6] Hoogerwaard EM, Bakker E, Ippel PF, Oosterwijk JC, Majoor-Krakauer DF, Leschot NJ, et al. Signs and symptoms of Duchenne muscular dystrophy and Becker muscular dystrophy among carriers in the Netherlands: a cohort study. Lancet 1999;353:2116–19.
[7] Moser H, Emery A. The manifesting carrier in Duchenne muscular dystrophy. Clin Genet 1974;5.
[8] Norman A, Harper P. A survey of manifesting carriers of Duchenne and Becker muscular dystrophy in Wales. Clin Genet 1989;36.
[9] Soltanzadeh P, Friez MJ, Dunn D, von Niederhausern A, Gurvich OL, Swoboda KJ, et al. Clinical and genetic characterization of manifesting carriers of DMD mutations. Neuromuscul Disord 2010;20:499–504.
[10] Brioschi S, Gualandi F, Scotton C, Armaroli A, Bovolenta M, Falzarano MS, et al. Genetic characterization in symptomatic female DMD carriers: lack of relationship between X-inactivation, transcriptional DMD allele balancing and phenotype. BMC Med Genet 2012;13:73.
[11] Preuße C, Goebel HH, Held J, Wengert O, Scheibe F, Irlbacher K, et al. Immune-mediated necrotizing myopathy is characterized by a specific Th1-M1 polarized immune profile. Am J Pathol 2012;181:2161–71.
[12] Allenbach Y, Benveniste O, Goebel H, Stenzel W. Integrated classification of inflammatory myopathies (2017):62–81.
[13] Allenbach Y, Leroux G, Suárez-calvet X, Preusse C, Gallardo E, Hervier B, et al. Dermatomyositis with or without anti-melanoma differentiation-associated gene 5 antibodies common interferon signature but distinct NOS2 expression 2016;186.
[14] Dalakas MC. Pathogenesis and therapies of immune-mediated myopathies. Autoimmun Rev 2012;11:203–6.
[15] De Bleecker JL, De Paepe B, Aronica E, de Visser M, Amato A, Benveniste O, et al. 205th ENMC international workshop: pathology diagnosis of idiopathic inflammatory myopathies part II 28-30 March 2014, Naarden, The Netherlands. Neuromuscul Disord 2015;25:268–72.
[16] Engel A, Arahata K, Emslie-Smith A. Immune effector mechanisms in inflammatory myopathies. Res Publ Assoc Res Nerv Ment Dis 1990;68:141–57.
[17] Allenbach Y, Mammen AL, Benveniste O, Stenzel W, Allenbach Y, Amato A, et al. 224th ENMC international workshop::: clinico-sero-pathological classification of immune-mediated necrotizing myopathies Zandvoort, The Netherlands, 14–16 October 2016. Neuromuscul Disord 2018;28:87–99.
[18] Allenbach Y, Arouche-delaperche L, Preusse C, Radbruch H, Butler-browne G, Champtiaux N, et al. Necrosis in anti-SRP and anti-HMGCR myopathies role of autoantibodies and complement. Neurology 2018;90:507–17.
[19] Wynn TA. Cellular and molecular mechanisms of fibrosis. J Pathol 2009;214:199–210.
[20] Villalta SA, Nguyen HX, Deng B, Gotoh T, Tidbal JG. Shifts in macrophage phenotypes and macrophage competition for arginine metabolism affect the severity of muscle pathology in muscular dystrophy. Hum Mol Genet 2009;18:482–96.
[21] Tidball JG, Villalta SA. Regulatory interactions between muscle and the immune system during muscle regeneration. Am J Physiol Regul Integr Comp Physiol 2010;298:R1173–87.
[22] Martinez FO, Helming L, Gordon S. Alternative activation of macrophages: an immunologic functional perspective. Annu Rev Immunol 2009;27:451–83.
[23] Murray PJ, Allen JE, Fisher EA, Lawrence T. Experimental Guidelines 2015;41:14–20.
[24] Schultze JL. Transcriptional programming of human macrophages: on the way to systems immunology. J Mol Med 2015;93:589–97.
[25] Murray PJ, Wynn T a. Protective and pathogenic functions of macrophage subsets. Nat Rev Immunol 2011;11:723–37.
[26] Murray PJ, Wynn T a. Obstacles and opportunities for understanding macrophage polarization. J Leukoc Biol 2011;89:557–63.
[27] Wynn TA. Fibrotic disease and the T(H)1/T(H)2 paradigm. Nat Rev Immunol 2004;4:583–94.
[28] Wilson M, Wynn T. Pulmonary fibrosis: pathogenesis, etiology and regulation. Mucosal Immunol 2009;2:103–21.
[29] Wynn TA. Integrating mechanisms of pulmonary fibrosis. J Exp Med 2011;208:1339–50.
[30] Wehling-Henricks M, Jordan MC, Gotoh T, Grody WW, Roos KP, Tidball JG. Arginine metabolism by macrophages promotes cardiac and muscle fibrosis in mdx muscular dystrophy. PLoS One 2010;5.
[31] Villalta SA, Rinaldi C, Deng B, Liu G, Fedor B, Tidball JG. Interleukin-10 reduces the pathology of mdx muscular dystrophy by deactivating M1 macrophages and modulating macrophage phenotype. Hum Mol Genet 2011;20:790–805.
[32] Villalta SA, Rosenthal W ML. Regulatory T cells suppress muscle inflammation and injury in muscular dystrophy. Sci Transl Med 2014;6(258):258 1–22.
[33] Villalta SA, Deng B, Rinaldi C, Wehling-Henricks M, Tidball JG. IFNγ promotes muscle damage in the mdx mouse model of Duchenne muscular dystrophy by suppressing M2 macrophage activation and inhibiting muscle cell proliferation. J Immunol 2014;71:3831–40.
[34] Soslow JH, Markham LW, Bryan Burnette W, Galindo CL, Feoktistov I, Raucci FJ, et al. Increased number of circulating CD8/CD26 T cells in the blood of duchenne muscular dystrophy patients is associated with augmented binding of adenosine deaminase and higher muscular strength scores. Front Pharmacol 2017;8:1–11.
[35] Kissel J, Burrow K, Rammohan K, Mendell J. Mononuclear cell analysis of muscle biopsies in prednisone-treated and untreated Duchenne muscular dystrophy. CIDD study group. Neurology 1991;41:667–72.
[36] Dobaczewski M, Chen W, Frangogiannis NG. Transforming Growth Factor (TGF)-ß signaling in cardiac remodeling. J Mol Cell Cardiol 2014;71:3831–40.
[37] Khalil H, Kanisicak O, Prasad V, Correll RN, Fu X, Schips T, et al. Fibroblast-specific TGF-β–Smad2/3 signaling underlies cardiac fibrosis. J Clin Invest 2017;127.
[38] Han A, Lu Y, Zheng Q, Zhang J, Zhao Y, Zhao M, et al. Qiliqiangxin attenuates cardiac remodeling via inhibition of TGF-β1/Smad3 and NF-κB signaling pathways in a rat model of myocardial infarction. Cell Physiol Biochem 2018;45:1797–806.

[39] Xiao Y, Ye J, Zhou Y, Huang J, Liu X, Huang B, et al. Baicalin inhibits pressure overload-induced cardiac fibrosis through regulating AMPK/TGF-β/Smads signaling pathway. Arch Biochem Biophys 2018:37–46.

[40] Bartekova M, Radosinska J, Jelemensky M, Dhalla NS. Role of cytokines and inflammation in heart function during health and disease. Heart Fail Rev 2018;23:733–58.

[41] Walcher T, Steinbach P, Spieß J, Kunze M, Gradinger R, Walcher D, et al. Detection of long-term progression of myocardial fibrosis in Duchenne muscular dystrophy in an affected family: a cardiovascular magnetic resonance study. Eur J Radiol 2011;80:115–19.

[42] Finsterer J, Stöllberger C, Freudenthaler B, de Simoni D, Höftberger R, Wagner K. Muscular and cardiac manifestations in a Duchenne-carrier harboring a dystrophin deletion of exons 12-29. Intractable Rare Dis Res 2018;7:120–5.

[43] Papa R, Madia F, Bartolomeo D, Trucco F, Pedemonte M, Traverso M, et al. Genetic and early clinical manifestations of females heterozygous for Duchenne/Becker muscular dystrophy. Pediatr Neurol 2016;55: 58–63.

[44] Yoon J, Kim SH, Ki CS, Kwon MJ, Lim MJ, Kwon SR, et al. Carrier woman of Duchenne muscular dystrophy mimicking inflammatory myositis. J Kor Med Sci 2011;26:587–91.